Faculty of Resource Science and Technology

SCREENING FOR ANTIMICROBIAL ACTIVITIES IN SOIL MICROBES ISOLATED FROM NANGA MERIT SITE 2

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A report submitted in partial fulfillment for the degree of Bachelor of Science with honours in Resource Biotechnology

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Declaration

I hereby declare that all the writing in this dissertation is from my own work except for some quotes which I have stated its source of origin.

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List of Abbreviations

CDA	Czapek Dox agar
^{0}C	Degree Celsius
%	Percent
µg	micro gram
µl	micro liter
FRST	Faculty of Resource Science and Technology
g	Gram
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimal Inhibitory Concentration
mg	Milligram
ml	milliliter
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NA	Nutrient Agar
NB	Nutrient Broth
nm	Nanometer
PBS	Phosphate Buffer Saline
PDA	Potato Dextrose Agar
pH	A measurement of the acidity or alkalinity of solution [p stands for "potenz" which means the potential to be while H stands for Hydrogen]

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Screening for Antimicrobial Activities in Soil Microbes Isolated from Nanga Merit Site 2

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ABSTRACT

A study was carried out on soil samples collected from Nanga Merit forest (Site 2) in the efforts to discover novel antibiotics produced by soil microbes. A total of 30 bacterial and 38 fungal isolates were successfully selected and colony purified. Antibiotic activity screening by using agar overlay technique followed by secondary screening have obtained one bacterial isolate (B17) and four fungal isolates (F10, F11, F14, and F26) that showed strong antibacterial activity against different strains of test bacteria. These test bacteria were *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, and *Enterobacter aerogenes*. Some synergistic effects were showed by fungal isolates (F2, F5, F17, F18, F20, and F21) grown on CDA when tested against *S. aureus*, *E. coli*, and *E. aerogenes*. Only one bacterial isolate (B17) and 12 fungal isolates (F2, F3, F5, F17, F18, F20, F21, F10, F11, F14, and F26) were selected for further antibiotics susceptibility testing and characterisation. Selected fungal isolates were cultivated on three types of culture media (PDA, CDA, and V8 Juice Agar). Only one extract (B17) was selected for MIC value determination. The potential of B17 to be further studied was showed by the small MIC value (<1.5625 mg/ml) and large inhibition size that was comparable to the positive control (5× dilutions of penicillin-streptomycin solution). Besides, extract of B17 also showed antifungal activity when tested against test fungus (*Fusarium* sp.). Besides, fungal isolates (F10, F11, F14, and F26) cultivated on PDA were also exhibited good antifungal activities when tested against the same test fungus. B17 was tentatively identified as *Phenylobacterium* sp. based on their physical appearances and biochemical tests results. Among the 11 fungi which were subjected to characterisation, seven isolates (F2, F3, F5, F17, F18, F20, F21, F10, F11, F14, and F26) and four isolates (F10, F11, F14, and F26) were tentatively identified as *Penicillium* spp. and *Streptomyces* spp., respectively. Further tests will be needed in order to determine the exact identity of these isolates.

Key words: novel, soil microbes, antibacterial activity, antifungal activity, MIC value

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ABSTRAK

Satu kajian telah dijalankan atas sampel tanah dari Hutan Nanga Merit (Kawasan 2) dengan usaha untuk mencari antibiotik baru yang dihasilkan oleh mikrob tanah. Terdapat 30 pencilan bakteria dan 38 pencilan kulat telah berjaya diplih dan dipurifikasi. Ujian penyaringan antibiotik dengan menggunakan teknik agar overlay diikuti dengan ujian penyaringan kedua telah memperolehi satu pencilan bakteria (B17) dan empat pencilan kulat (F10, F11, F14, dan F26) yang mempunyai aktiviti anti-bakteria yang kaut terhadap empat jenis bakteria ujian iaitu Staphylococcus aureus, Salmonella typhi, Escherichia coli, and Enterobacter aerogenes. Kesan sinergistik terhadap S. aureus, E. coli, and E. aerogenes dapat dikesan bagi pencilan kulat (F2, F3, F5, F17, F18, F20, dan F21) yang dikultur atas CDA. Hanya satu pencilan bakteria (B17) dan 11 pencilan kulat (F2, F3, F5, F17, F18, F20, F21, F10, F11, F14, dan F26) telah dipilih untuk ujian penyaringan lanjutan dan pencirian. Pencilan kulat yang telah diplih dikulturkan di atas tiga jenis media iaitu PDA, CDA, dan Agar Jus V8. Penentuan nilai MIC hanya dilakukan untuk ekstrak B17 sahaja. Potensi untuk menjalankan kajian lanjutan terhadap B17 telah ditunjukkan melalui nilai MIC yang kecil (<1.5625 mg/ml) dan kemampuannya untuk menghasilkan zon perencatan yang setanding dengan kawalan positif (5× cecair penicillin-streptomycin). Ekstrak B17 juga menujukkan aktiviti anti-kulat dengan merencat pertumbuhan kulat ujian (Fusarium sp.). Selain itu, pencilan kulat (F10, F11, F14, dan F26) yang dikultur atas PDA juga mempunyai aktiviti anti-kulat yang kuat apabila diuji dengan kulat ujian yang sama. B17 dikenalpasti secara sementara sebagai Phenylobacterium sp. berdasarkan ciri-ciri fizikal dan ujian biokimia yang telah dijalankan. Bagi kulat pula, terdapat tujuh pencilan (F2, F3, F5, F17, F18, F20, F21, F10, F11, F14, dan F26) dan empat pencilan (F10, F11, F14, dan F26) masing-masing dikenalpasti secara sementara sebagai Penicillium spp. dan Streptomyces spp.. Ujian lanjutan diperlukan untuk mengenalpasti identiti sebenar pencilan-pencilan tersebut.

Kata kunci: baru, mikrob tanah, aktiviti anti-bakteria, aktiviti anti-kulat, nilai MIC

1.0 Introduction

Antibiotics are substances use to against infection caused by bacteria. Antibiotics act by inhibiting or destroying bacterial cells (Duerden *et al*., 1993) that cause certain disease. In nature, antibiotics are secondary metabolites produced by bacteria (Demain, 2000) in order to maintain their niche and territory. Only limited groups of microorganisms are responsible for the sources of clinically useable antibiotics. As stated by Cooke and Gibson (1983), only those that have an effect on bacterial cells but not the host cells like human are categorised as useful antibiotics. Besides, there are only limited numbers of antifungal antibiotics that can be used to treat fungal infections (Rusell, 1977).

According to Tortora *et al*. (2007), antibiotics can be found easily but only few are useful. Hence, concerted efforts have been carried out by many scientists in order to screen for novel antibiotic producing microbes (Oskay *et al*., 2004). Through their efforts, many antibiotics have been successfully discovered in order to combat pathogenic bacteria that cause diseases. However, the emergence of new diseases and reemergence of multiple-antibiotic resistant pathogens have rendered the existence used antibiotics ineffectively. This problem has spurred the needs for the discovery of new antibiotics (Roberts, 1998).

Among the diverse sources of antibiotics, soil is the most important target for scientists in the discovery of novel antibiotics. According to Dulmage & Rivas (1978), soil microorganisms have continually been screened for their useful biological active metabolites such as antibiotics since long ago. Therefore, this study is an attempt to discover novel antibiotics from soil samples from remote area (Nanga Merit forest). These soil samples were chosen for analysis because the likely hood of isolating novel antibiotics producing soil microorganisms from this area is high.

Hence, the objectives of this study are:

1. To isolate and characterise the antibiotic producing soil microbes.

2. To determine the antimicrobial properties of soil microbes towards test microorganisms.

3. To determine the minimal inhibitory concentration (MIC) values of the isolated antibiotics.

2.0 Literature Review

2.1 Pathogenic microorganisms

Bacteria, fungi, viruses, and protozoan are microorganisms that are either harmless or can cause diseases. Only those that have pathogenic characteristics can cause infections and the capabilities of pathogens to cause infections differ in some degrees (Gilbert, 1977). According to Duerden *et al*. (1993), pathogens which infect human may come from either exogenous or endogenous sources. Bacterial infections usually can be cured through antibiotics but the emergence of multi-drug resistance pathogens has created obstacles in treating diseases.

2.2 Multiple-drug resistant pathogenic microorganisms

The presences of antibiotics supposed to suppress the infection of pathogenic microorganisms (Hamilton-Miller, 2004). However, the misuse and widely use of antibiotics for medication and animal breeding in inappropriate dosage have creating antibiotics resistant pathogens (Lynch *et al*., 2004; Theuretzbacher, 2009). This is either due to the mutations that occur in bacterial chromosome or development of antibiotics resistant strains through the exchange of genetic materials between bacterial cells (Cooke & Gibson, 1983; Roberts, 1998; Cirz *et al*., 2005). Besides, according to Chung *et al*. (2008), the emergences of multi-drug resistant bacteria are also due to inheritance factors. Hence, the discoveries of new antimicrobial drugs and renewed derivatives of the previous antibiotics may be useful for a period of time and resistance will develop shortly (Roberts, 1998; van der Waaij *et al*., 2000).

Some of the common examples of these multiple-drug resistant pathogenic microorganisms are the methicillin (and multidrug) resistant *Staphylococcus aureus* (MRSA), multiple-drug resistant (MDR) enterococci, and multi-drug resistant

Streptococcus pneumoniae (Hart, 1998; Huycke *et al*., 1998; Xu *et al*., 2009). These multidrug-resistant pathogens can cause severe diseases especially in patients who are immunocompromised and for those who stays in intensive care unit (ICU) (Weber *et al*., 2007). Besides bacteria, microbes like fungus such as *Candida albicans* can also confer resistance towards different antibiotics. As stated by Gulshan and Rowley (2007), *C. albicans* has been involved in broad studies by researchers due to its property that can resist different antimicrobial drugs.

The number of multi-drug resistant pathogenic microbes has increased over time and there are only limited therapeutic drugs that are applicable to combat these pathogens (Roberts, 1998; Demain & Sanchez, 2009). Therefore, there is a need for continuous discoveries of new antibiotics in order to make treatments under antibiotics remain effective (Roberts, 1998; van der Waaij *et al*., 2000).

2.3 Antibiotics

Antibiotics are substances produced by microbes which can be used to inhibit the growth of other microorganisms at low concentration (Rusell, 1977). In their natural habitats, bacteria utilise the antibiotics they produce as protective substances by preventing the invasion of other bacterial species. Protection is not the only function of antibiotics. Hence, according to Linares *et al*. (2006), antibiotics also act as signaling molecules that bacteria use as a means of communication between cells.

Antibiotics can be classified according to their mode of actions. Antibiotics are classified as broad-spectrum antibiotics when they have the ability to affect a wide range of Gram-positive and Gram-negative bacteria while antibiotics that only effective towards certain group of bacteria are known as narrow-spectrum antibiotics. Several mechanisms of actions of antibiotics have been discovered by scientists. These actions include the

inhibition of cell wall, protein and nucleic acids synthesis (Lambert, 1977; Brooks *et al*., 2001; Tortora *et al*., 2007).

There are three important groups of microorganisms which are responsible for the production of antibiotics. These are the Gram-positive rod shape bacteria such as *Bacillus*, actinomycetes, and fungi such as *Cephalosporium* and *Penicillium* (Tortora *et al*., 2007). Actinomycetes are the Gram-positive bacteria that contribute most of the clinically use antibiotics and as stated by Oskay *et al*., (2004), the discovery of new biological metabolites particularly useful antibiotics from actinomycetes need a vast amount of isolates. Majority of the antibiotics that have been identified and presently in use are isolated from the bacteria under the genus of Streptomycetes. Examples of these antibiotics are tetracycline and streptomycin.

As compared to antibacterial agents, the development of antifungal agents is not achieved high advancement. This is because the lethal targets for infectious fungal species are hard to be identified due to the similarity of metabolic pathways possessed by them and their host, mammals since both are classified as eukaryotes (Imada & Hotta, 1992). Griseofulvin and nystatin are examples of antifungal antibiotics that can be used to cure infections caused by *Trichophyton* and *C. albicans*, respectively (Russell, 1977).

2.4 Sources of natural occurring antibiotics

Antibiotics-producing microbes can be isolated from different sources such as soil and marine microbes, endophytes, lichens, and even animals. According to von Bubnoff (2006), some scientists are continually searching for novel antibiotics producing microorganisms from extraordinary places such as deep sea mud and seaweeds. In addition, endophytes which inhabit in higher plants also become one of the important

sources of antibiotics which are effective against different types of pathogens (Strobel & Daisy, 2003).

An early study conducted by Burkholder *et al*. (1944) had shown that lichens have high potential to produce useful antibiotics. In their study, of the 42 lichens species, 64.29% of the species did show active antimicrobial activity against *S. aureus* and *B. subtilis*. Besides, animals also become target for scientists to search for novel antibiotics. According to Margavey *et al*. (2004), a broad spectrum of antibiotics, squalamine has been successfully isolated from the stomach tissues of dogfish shark *Squalus acanthias*.

As discussed above, there are many sources where antibiotics can be discovered but soil still remains the most important target for most researchers in their efforts to discover novel antibiotics that have pharmaceutical values. This is because many microbes especially bacteria that reside in soil have the ability to produce biologically active secondary metabolites such as useful antibiotics.

2.5 Antibiotic producing soil microbes

Soil is a reservoir where most antibiotics producing microbes and their secondary metabolites can be found. Actinomycetes are Gram-positive bacteria which form spore and filamentous and they are the most important group of antibiotic producing soil microorganisms since they contribute to 75% of the identified products which are widely used in clinical applications (Oskay *et al*., 2004; Ceylan *et al*., 2008). According to Demain (2000), there will be about 500 antibiotics from actinomycetes continually being discovered each year.

As reported by Oskay *et al*. (2004), of the 50 isolates that they obtained from actinomycetes, 34% of the isolates did produce antibiotics. Besides, from the recent research work conducted by Ceylan *et al*. (2008), they also reported that 15 isolates

obtained from the genus of Streptomycetes showed the capability of producing antibacterial substances towards both Gram-positive and Gram-negative bacteria which resistance to different antibiotics. Streptomycetes are categorized in the family of Streptomycetaceae (Anderson & Wellington, 2001) and different species of bacteria classified under this genus contribute mostly to the useful biologically active substances such as antibiotics that have been authorized.

Besides, the ability of *Streptomyces* to act as useful biological control agents in retarding the growth of pathogenic fungi which infected plants also has been reported by many researchers. These pathogenic fungi may either arise from soil or air (Oskay, 2009). In addition, the current research conducted by Oskay (2009) has discovered that there was a novel strain of *Streptomyces* assigned as *Streptomyces* sp. KEH23 that has a high potential to produce useful antibiotics which can actively against pathogens that infected plants and human being.

Streptomycetes can be widely found in both terrestrial and aquatics environments especially where nutrients are highly abundant such as in the soil, hay, and composts (Locci, 1989). Besides, there are several factors which can influence the distribution of streptomycetes which including the temperature, moisture, pH, and climate (Williams *et al*., 1972b; Williams, 1978 in Locci, 1989).

Besides Streptomycetes, other Gram positive soil bacterium such as *Rhodococcus* has also been identified to have high potential of producing useful antibiotics when they are in stress condition. As reported by Robson (2008), a research team led by Kazuhiko Kurosawa has isolated the amino glyceride antibiotic, rhodostreptomycin produced by *Rhodococcus*. This antibiotic is effective against many types of test microorganism which including the hardy strain Streptomycetes.

3.0 Materials and Methods

3.1 Sources of materials

3.1.1 Soil samples

A total of 10 soil samples (Table 1) of 20 grams each were obtained from FRST Soil Laboratory. Each soil sample was scooped from a larger sample volume and was put into respective plastic bag under aseptic condition. These samples were collected from Nanga Merit forest (Site 2) by the Zoonosis Research Group members for FRST. The plastic bag contained the soil samples were labeled specifically and kept at 4^0C in Virology Laboratory before they were analysed.

Table 1: Soil sample designation according to sites and depths

Depth where soil samples are taken (cm)	Soil samples				
0-20	S1 (i)	S2 (i)	S3 (i)	S4 (i)	S5 (i)
20-40	S1 (ii)	S2 (ii)	S3 (ii)	S4 (ii)	S5 (ii)

3.1.2 Solutions and media

Phosphate buffer saline (PBS) (pH 7), Nutrient Agar (NA) (Merck, USA), Nutrient Broth (NB) (Merck, USA), Potato Dextrose Agar (PDA) (Oxoid), soft Nutrient Agar (Oxoid), V8 Juice Agar (Atlas, 2006), Czapek Dox Agar (CDA) (Merck, USA), Mueller-Hinton Broth (MHB) (Oxoid), and Mueller-Hinton Agar (MHA) (Oxoid) were used in this study in order to cultivate bacterial and fungal colonies needed in this study. These media were also used in the antimicrobial test and during the determination of minimum inhibitory concentration (MIC) value for selected methanol extract. Besides, Methanol solvent was used to extract potential secondary metabolites from selected bacterial and fungal isolates.

3.1.3 Test microorganisms

In this study, four species of test bacteria were used for the preliminary testing. They were *S. aureus* (Gram-positive bacterium), *Escherichia coli* (Gram-negative bacteria), *Enterobacter aerogenes* (Gram-negative bacteria), and *Salmonella typhi* (Gram-negative bacteria). These test bacteria were obtained from stock culture at Virology Laboratory, UNIMAS.

3.2 Preparation of soil samples and plating

One gram of soil was weighted and put into 15 ml Falcon tube to which 10 ml of sterile Phosphate Buffer Saline (PBS) buffer at the pH 7.0 was subsequently added. The mixture of soil and PBS was homogenised by using a Vortex mixer. The tube was then left for 1 hour to allow the large soil particles to completely settle. Subsequently, 100 µl of the supernatant from the soil suspension was pipetted and spread over four NA plates and four PDA plates, respectively, by using sterile cotton swabs. Each plate was labeled specifically. The plates were left at room temperature (26^0C) for five days in order for the bacteria and fungus to grow. After 5 days, the amount of growth, that is the number of bacterial or fungal colonies was calculated and recorded. The plates were kept at 4^0C for two days in order to delay the growth of soil microorganisms before preliminary antibiotic screening was carried out.

3.3 Antibiotic activity screening by agar overlay technique

3.3.1 Cultivation of test bacteria and preliminary selection

Test bacteria were cultured in 3 ml of NB at 37^0C for 18 to 24 hours the day before use (Fankhauser, 2005). The turbidity of the test bacteria was determined by using spectrophotometer at wavelength of 520 nm. The optical density (OD) value was adjusted

to 0.6 (J-Nkanga & Hagedorn, 1978). Soft NA (0.75%) was prepared and kept at 50^0C until use. After determination of the OD value, 100 µl of test bacteria was pipetted and mixed well with 2ml of 0.75% soft agar (Fankhauser, 2005) which was kept at 50^0C. The mixture of the test bacteria and soft NA was vortexed for 10 to 20 seconds in order to mix. The mixture of soft agar was then overlaid on the NA plates and PDA plates grown with soil microorganisms from plating before the soft NA solidified. Four empty plates of NA and PDA were overlaid with the test bacteria and were used as controls in this study. The plates were kept at room temperature (26^0C) and observation for the formation of inhibition zone was carried out for every 24 hours and 48 hours.

3.3.2 Selection and isolation of soil microorganisms

Soil microorganisms were selected and isolated based on two approaches as stated below:

(I) After inoculation of soil microbes

Soil microorganisms that showed activity against other soil bacteria or fungus in the same plate were selected and isolated in order to obtain pure culture.

(II) From preliminary selection

Soil microorganisms that inhibited the growth of test bacterial by producing inhibition zone were selected and isolated in order to obtain pure strain of that particular microorganism. Each isolated soil microorganisms were assigned with specific codes. The pure strains of microorganisms were stored as stock culture in either slant NA or PDA at 4^0C for further usage.

3.4 Secondary screening

3.4.1 Secondary screening for bacterial isolates via spot inoculation method

Pure bacterial isolates selected after inoculation of soil microbes and preliminary selection were cultivated on NA via spot inoculation method. Each NA plate was spot inoculated with nine pure bacterial isolates and was incubated at room temperature (26^0C) for four days. After that, 2 ml of 0.75% soft NA inoculated with test bacteria was overlaid onto the NA plate containing the pure bacterial isolates. Soft NA which has been seeded with test bacteria was vortexed for 10 to 20 seconds in order to mix before overlaid on the basal medium. Four empty plates of NA were also overlaid with soft agar containing the different species of test bacteria and were used as controls in this experimentation. These plates were incubated at room temperature (26^0C) and zone of inhibition was observed after 24 hours of incubation period.

3.4.2 Secondary screening for fungal isolates via agar overlay technique

Pure fungal isolates which have been selected after inoculation of soil microbes and preliminary selection were cultivated on two different media (CDA and V8 Juice Agar) with five fungal isolates per plate. After incubation at room temperature (26^0C) for 4 days, these fungal isolates which grew on different medium were subjected to secondary screening via agar overlay technique, whereby 2 ml of 0.75% soft NA which has been seeded with test bacteria was overlaid onto the growing fungal isolates. Besides, as in primary screening and spot inoculation methods, four empty CDA and V8 Juice Agar plates which were overlaid with inoculated soft agar were used as controls in this experiment. These plates were incubated at room temperature (26^0C) and zone of inhibition was observed after 24 hours of incubation period.

3.5 Antibacterial activity of soil microbes extracts

3.5.1 Cultivation of selected bacterial and fungal isolates

Bacterial and fungal isolates that have showed good antibacterial activities during the secondary screening were selected and cultivated on different culture media with 3 plates per isolate. These culture media were NA (for bacterial isolates), PDA, CDA, and V8 Juice Agar (for fungal isolates).

Selected bacterial isolates were first inoculated into NB and incubated at room temperature (26^0C) for 3 days. After that, the inoculated NB was swabbed onto NA plates with sterile cotton swabs and the plates were incubated at 26^0C for another 3 days. Similarly, selected fungal isolates were cultivated on different media (PDA, CDA, and V8 Juice Agar) at room temperature (26^0C) until they reached full plate. All plates were placed inverted in secluded area with the covers removed for drying.

3.5.2 Extraction of secondary metabolites

After drying, the agars were grinded separately with mortar and pestle before they were immersed with methanol solvent in conical flasks. After 4 days of immersion, the solvent was filtered and the agar residual from the previous filtration was re-immersed in new methanol solvent for another one day before further filtration was carried out.

The methanol solvent which might contain the potential secondary metabolite was concentrated by using rotary evaporator at 40^0C. The concentrated secondary metabolites were then placed in a desiccator for another 3 days for the removal of excess moisture. Then, the crude methanol extracts were collected, weighted, and kept in 1.5 ml eppendorf tube at 4^0C for further usage. The eppendorf tubes were covered with aluminium foils in order to prevent the crude methanol extracts to have direct contact with light source.